JOURNAL OF MEDICAL VIROLOGY

VOLUME 23, 1987

Arie J. Zuckerman

Editor

Alan R. Liss, Inc., New York

Contents

Volume 23, Number 1 September 198

Journal of Medical Virology 23:51–66 (1987)

Isolation Frequency and Growth Properties of HIV-Variants: Multiple Simultaneous Variants in a Patient Demonstrated by Molecular Cloning

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The biological properties and efficiency of isolation of different HIV (LAV/HTLV III, ARV, and AAV) subtypes were evaluated by recovering and growing HIV on fresh peripheral human lymphocytes. Cultures for virus isolaton were performed from more than 180 German AIDS, ARC, LAS, and virus-exposed asymptomatic patients. The virus isolation rate depended on the state of health of the patients being close to 80% in AIDS patients, 30–40% in ARC/LAS patients, and lower in asymptomatic HIV seropositive patients.

The cytopathic effects of the HIV isolates obtained on lymphocyte-cell cultures ranged from no effect to marked syncytia formation and cytopathogenicity. Marked differences were also observed in the replication rate of the various isolates. These properties were stable in all in vitro passages of the viruses performed so far and allowed to tentatively define four subtypes of HIV. In the majority of AIDS cases with neurological symptoms well-growing strains were obtained from peripheral blood, while all but two isolates from the cerebrospinal fluid of the same patients grew remarkably slowly and to only low titres on lymphocytes, suggesting that selection of variants for growth at specific sites of the body occurs.

For one of the most cytopathogenic strains the influence of several variables of culture conditions (cell type, corticosteroids, IL-2, and polybrene) on virus replication was studied. Apart from polybrene, all parameters strongly influenced replication.

Accepted for publication March 16, 1987.

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The restriction analysis of several DNA clones derived from one HIV isolate revealed marked restriction site polymorphism between these clones. This result demonstrated that at the time of virus isolation the patient carried at least four different variants. Multiple variants can thus infect a patient or arise within a patient.

Key words: HIV-variants (LAV/HTLV III), growth conditions, stimulation, tropism, pathogenicity, molecular cloning

INTRODUCTION

From the beginning, infections with human immunodeficiency virus (*HIV = LAV/HTLV III, AAV, and ARV) have confronted the scientific community with many questions. Among these were the questions why so very variable incubation times and clinical patterns were observed and why this retrovirus was so highly pathogenic.

As more isolates of HIV were obtained it became apparent that HIV is not a single well-defined virus type but a large family of highly divergent subtypes [Wain-Hobson et al, 1985; Hahn et al, 1985; Muesing et al, 1985; Rübsamen-Waigmann et al, 1986a,b]. Variations were found not only in restriction patterns or nucleotide sequences but also in biological properties of the viruses, like replication rate and cytopathic effect on lymphocytes [Rübsamen-Waigmann et al, 1986a,b]. It also became apparent that not all HIV subtypes grow well on lymphocytes [von Briesen et al, 1986]. Cells of the monocyte/macrophage lineage were found to be important in vivo target cells of some HIV strains [Nicholson et al, 1986; Salahuddin et al, 1986; Gartner et al, 1986a,b].

Recently, two HIV-related virus types were isolated in West Africa, one from AIDS patients [Clavel et al, 1986a] and one from healthy individuals [Kanki et al, 1986]. These isolates appeared similar to each other but only distantly related to the original isolates of LAV/HTLV III (=HIV I). They were called LAV II (=HIV II) and HTLV IV, respectively. Sequence determination of one LAV II isolate revealed a fairly close relationship to the SIV family of monkey viruses [Clavel et al, 1986b]. Strong restriction site polymorphism was found.

In view of this striking heterogeneity of HIV it may be assumed that the wide spectrum of incubation times and clinical manifestations of HIV infections may be related to the particular virus strain infecting a patient and/or arising within a patient during persistent infection.

In this communication we describe our observations on cultures for virus isolation from more than 180 patients. Isolation frequency was found to depend on the state of health of the patients. In addition, the biological properties of the variants isolated depended on the material from which they were recovered (blood or cerebrospinal fluid, respectively). Since their biological properties were stable in all in vitro passages, the viruses were tentatively grouped into four classes. One highly cytopathic strain was chosen to study several parameters, which are important for virus isolation and propagation of HIV in vitro.

Molecular cloning and restriction analysis of the cloned HIV DNA from one of the cytopathic isolates from peripheral blood revealed that multiple HIV variants coexisted in one and the same patient at the time of virus isolation.

*Abbreviations: HIV, human immunodeficiency virus (=LAV/HTLV III, ARV, AAV); SNF, supernatant fluid; CPE, cytopathic effect; CSF, cerebrospinal fluid; RT, reverse transcriptase; PBL, peripheral blood lymphocytes; IL-2, interleukin-2 = T cell growth factor, TCGF; EM, electron microscopy; pi, post infection.

MATERIALS AND METHODS

HIV Isolation From AIDS and ARC Patients

HIV was isolated from the patients' peripheral blood lymphocytes (recovered from 5–20 ml of heparinized blood) and cerebrospinal fluid (0.5–1 ml) as previously described [Rübsamen-Waigmann et al, 1986a,b; von Briesen et al, 1986].

Peripheral Blood Lymphocyte (PBL) Culture and Propagation of HIV (Strain HIV_{D34})

HIV was propagated in normal peripheral blood lymphocytes isolated from newborn umbilical cord blood. Lymphocytes were separated and cultured as described elsewhere [Boyum, 1968; Rübsamen et al, 1986a]. To obtain high virus titres (10^6 infectious units/ml, as determined by end-point titration) we used SNF from strain HIV_{D34} stored at −70 °C (titre after thawing: 10^3–10^4 infectious units/ml) and inoculated 1 ml of this SNF into 5 ml of a suspension of freshly stimulated normal cord blood lymphocytes (about 5×10^6 cells). On day 3–4 after inoculation virus titre in the SNF of this culture was about 10^6 infectious units/ml. In some cases (see Results), hydrocortisone (Sigma, Deisenhofen, FRG) or dexamethasone (Merck Sharp and Dohme, München, FRG) was included in the cultures.

Reverse Transcriptase (RT) Assay

Reverse transcriptase activity was assayed in the cell-free supernatants of infected and uninfected (control) PBL cultures [Rübsamen et al, 1986a] after ultracentrifugation.

Electron Microscopy (EM)

HIV_{D34} [=AAV_{D34} Rübsamen-Waigmann et al, 1986a] infected cells (PBL) were grown as suspension cell cultures in 50-ml plastic flasks (Nunc) and harvested for thin section EM on the third day after infection showing fully developed CPE and 2×10^6 infectious units/ml [Gelderblom et al, 1987; Venable and Coggeshall, 1965; Simionescu and Simionescu, 1976].

Genetic Characterization of HIV Strains

Molecular cloning of HIV-DNA fragments. 3×10^7 cells (PBL) were infected with HIV_{D31} [=AAV_{D31}, Rübsamen et al, 1986a]. On day 3 after inoculation the cells were harvested by the proteinase K/EDTA/SDS procedure (HIV_{D31}-DNA) [Maniatis et al, 1982]. The purified HIV_{D31}-DNA was digested by SstI. The cloning work was done under L2/B2 conditions using Escherichia coli DP50/lambda gt · WES as a EK2-host/vector system [Leder et al, 1977].

Lambda gt·WES-DNA was first treated with ligase to protect the cohesive ends and then cleaved by SstI. The DNA was treated with calf intestine phosphatase (Boehringer, Mannheim, FRG) in order to avoid self-ligation and to avoid the religation of the stuffer fragment; 2 μg of this lambda gt·WES vector DNA were ligated with 0.5 μg of SstI digested HIV_{D31}-DNA and packaged in vitro. Plating of transformed E. coli DP50 cells was performed on NZY plates (BRL, Freiburg, FRG) supplemented with thymidine (40 μg/ml) diaminopimelic acid (100 μg/ml) (Sigma, Deisenhofen, FRG), and 0.1% maltose (Merck, Darmstadt, FRG).

500,000 plaques were screened on nitrocellulose filters (Schleicher and Schüll BA 85) with a mixture of nick translated lambda BH5/BH8 inserts (5×10^7 cpm/μg) under moderate stringency. Hybridization was done for 24 hours at 42°C in 50% formamide, 5×SSPE, 5× Denhardt, 100 μg/ml salmon sperm DNA, and 300,000 cpm/ml of the probe. The filters were washed twice in 0.1 × SSC, 0.1% SDS at room temperature for 15 minutes and twice in 0.1 × SSC, 0.1% SDS at 42°C for 15 minutes. Nine positive plaques were picked and plaque-purified twice. Lambda DNA was isolated from five of these individually and characterized by Southern blotting.

Subcloning of HIV_{D31}-Lambda Clones Into Plasmid Clones and Restriction Mapping. The DNA inserts of five positive lambda clones (lambda 10.2, lambda 1.1, lambda 4.1, lambda 8.1, and lambda 2.1) were prepared by isolation and purification on Nucleogen-4000 HPLC columns (DIAGEN, Düsseldorf, FRG) and inserted into pTZ18R vector DNA (Pharmacia, Freiburg). The corresponding subclones pTZ31D, pTZ31G, pTZ31K, pTZ31L, and pTZ31H contained all of the five inserts in antiorientation to the T7 phage promoter. The plasmid DNAs were mapped with the enzymes SstI, HindIII, PstI, SphI, XbaI, Bg1II, BglI, KpnI, EcoRI, SalI, and BamHI.

RESULTS

Efficiency of HIV-Isolation and Characterization of the Types of Virus Strains

The efficiency of virus isolation from the patients studied so far is given in Table I. The isolates were characterized as HIV related as previously described [Rübsamen-Waigmann et al, 1986a].

While our isolation procedure yielded virus from peripheral blood in most AIDS cases and positive isolation was reproducible each time when it was attempted from a particular patient, it was not always possible to grow virus from pre-AIDS cases with clinical symptoms. The yields were even lower in asymptomatic carriers. These results were also reproducible in repeated attempts of virus isolation for a given asymptomatic patient as long as his health status did not deteriorate. It thus appeared that at least in the periphery many asymptomatic and LAS patients harboured very little virus-producing cells. We did, however, observe several cases where virus was readily isolated at a later time when such patients showed a marked decrease in their total T4 lymphocytes and in their T4/T8 ratio.

The characterization of the isolates recovered with respect to biological properties was based on three criteria: a) time required for reaching maximum RT activity or titre, b) maximal RT activity reached, and c) type of morphological changes induced by the virus strains in lymphocyte cultures. The classification of our isolates with respect to these criteria and the frequency of isolation of the biologically distinct types are given in Table II.

TABLE I. Efficiency of Virus Isolation and State of Health of the Patients

Isolation from blood[a]		Isolation from CSF[b]	
State of health	Positive isolation (%)	State of health	Positive isolation
AIDS	80	AIDS, neurological symptoms	7/16[c] (44%)
LAS, T4/T8 <0.5	50–70	LAS, neurological symptoms	4/11[d] (36%)
LAS, T4/T8 >0.5	30–40		
Asymptomatic, seropositive	20–30	Asymptomatic, seropositive	Not done

[a]The data are derived from 180 lymphocyte cultures from peripheral blood. In about 10% of the cases repeated cultures of specimens from the same patients were carried out over a period of up to 1 yr.
[b]Details of the clinical data are given in Rübsamen-Waigmann et al, 1986b.
[c]Out of these, two isolates grew well (type 1 viruses, see Table II), all others were type 4.
[d]All isolates were type 4 (see Table II).

TABLE II. Biological Properties of the HIV Strains Isolated

Type number	a) Replication to maximum RT or titre within (days)	b) Maximal RT in supernatant cpm/ml[a]	c) CPE	Frequency of isolation from: Blood	CSF
1	2–3	50,000–1,000,000	Clear syncytia, involving 20–100 cells	40/180	2/27
2	1–2	50,000–1,000,000	Giant, clear syncytia involving the whole colony	3/180	0/27
3	5–7	50,000–1,000,000	No syncytia, hardly cytopathic or non cytopathic	10/180[b]	0/27
4	7–21	3,000–10,000	Few syncytia involving less than ten cells, syncytia are often turbid	15/180	9/27

[a]The negative control (supernatant from uninfected cells, pelleted and assayed in the same way as the SNF from infected cells) had 900–2,000 cpm/ml.
[b]These include 6/6 isolates from hemophiliac AIDS patients.

Cytopathogenic viruses with efficient replication on PBL (types 1 and 2, Table II) developed highest particle production (measured by RT-activity: 50,000–1,000,000 cpm/ml SNF), as well as maximum syncytia formation, in up to 3 days when 5 ml of PBL culture suspension (about 1×10^6 cells/ml) were inoculated with about 100,000 RT-cpm. Type 1 viruses were most frequently observed. The very cytopathic viruses (type 2) formed striking syncytia in the cultures within 12–24 hours using the inoculum size described above. Interestingly, viruses with still good replication

but little or no CPE (type 3) were often obtained from hemophiliacs (6/6 patients, Table II).

All of the type 1–3 viruses could be grown to high titres and RT-values by enrichment through subcultures, if the primary culture contained low amounts of the viruses. In contrast, viruses with bad replication on PBL (type 4, Table II) needed 7–21 days until the cultures became RT-positive and could not be grown to higher titres by subcultures. These virus strains, in some cases, were even lost upon further subcultures on PBL. They all showed hardly any CPE in the infected cultures. It appears noteworthy that such types of viruses were obtained more frequently from CSF than from blood [see also Rübsamen-Waigmann, 1986b]. Furthermore, when virus isolation was carried out simultaneously from the blood and CSF of the same patient, the viruses obtained from the periphery frequently grew much better than the ones from the CSF. It thus appears that virus variants differ in their ability to replicate on lymphocytes (and probably monocytes/macrophages) and that not all variants that are present in the periphery of a patient reach the nervous system.

Characterization of a Type-2 Virus and Investigation of Parameters Influencing Growth In Vitro

For the prototype of HIV, LAV/HTLV III_B, several viral genes have been defined, which are believed to regulate its replication. It is conceivable that the viruses with differing replication potential isolated in this study might differ in the function of these genes. An isolate of the best replicating and most cytopathic strain of HIV (type 2) was therefore chosen to determine how culture conditions influenced its replication.

The virus chosen for these experiments, HIV_{D34}, displayed all typical morphological features of HIV upon electron microscopy (Fig. 1): It budded from the cell membrane (Fig. 1a,b) taking with it the lipid bilayer of the plasma membrane that surrounded the mature virus particles. These were characterized by an elongated core with "salt-seller morphology" (Fig. 1c, left particle), which, when cut across, was found to lie asymmetrically in the particle (Fig. 1c, right particle). To the virus membrane fuzzy structures were attached, which most likely represented the viral envelope glycoproteins. Other features characterizing the isolate HIV_{D34} as being related to the prototype LAV/HTLV III_B were hybridization in Southern blots under medium stringency with the probes lambda BH 5/8 [Rübsamen-Waigmann et al,

Fig. 1. Thin section electron microscopy of HIV_{D34} particles, grown in peripheral lymphocytes. Budding (a,b) and cell-released "mature particles" (c) are shown (see text). ×120,000. Bar, 100 nm.

1986a] and strong immunological reactions with positive anti-HTLV III_B reference sera (immunofluorescence, Western blot, data not shown).

Because of its strong cytopathic effect leading to rapid formation of giant cells, the virus titres of HIV_{D34} could easily be assessed by titration on PBL and direct microscopic inspection. Virus titre, RT-activity, and formation of CPE in a culture infected with low doses of virus were determined in parallel. The production of infectious virus as judged by titration (Fig. 2a) showed a very sharp rise and fall, being maximal only on day 4. In contrast to the production of infectious virus, which differed from the preceding day by a factor of 1,000 and from the following day by a factor of 100, particles with RT-activity were observed over a longer period of time and RT-values dropped only by a factor of 2 on day 7, where hardly any infectious virus was left. It thus appears as if the infectivity of free HIV_{D34} particles is fairly unstable under the conditions of cell culture. When a known amount of infectious virus was incubated in growth medium without cells at 37°C and titrated after various periods of time, infectivity dropped by about a factor of 10 within 24 hours.

CPE became detectable within 48 hours pi resulting in large clear syncytia. It progressed on day 3 showing the condensed nuclei within the syncytia (Fig. 2c). The clear cytoplasm of the syncytia burst on day 4 leaving behind several hundred condensed nuclei and bizarre structures (Fig. 2d) with brownish appearance.

Since we had observed that virus isolation was much easier using cord cells as opposed to established T-cell-lymphoma lines like H9 [Popovic et al, 1984] or B-cell-lymphoma lines like FR 8 [Montagnier et al, 1984], the cord lymphocytes used in the experiment of Figure 2 were compared to the T-cell-lymphoma line HUT 78 [Gazdar et al, 1980] for susceptibility to HIV_{D34}. When 10^6 infectious units of virus were inoculated into 5×10^6 HUT 78 cells (kept without addition of IL-2), CPE developed after 1 day. In contrast to cord cells, the virus production in this culture, however, remained low (10^2–10^3 infectious units/ml as opposed to 10^6 infectious units/ml in the cord cell culture (Fig. 2) and 10,000–20,000 RT-cpm/ml SNF). Furthermore, the culture was not killed by the infection but grew as a permanently virus-producing line for now 12 months (HUT $78/34_3$).

HUT 78 cells are tumor cells derived from a patient with CTCL (cutaneous T-cell lymphoma, mycosis fungoides) and thus are independent of exogenous IL-2 [Gazdar et al, 1980; Gootenberg et al, 1981]. They can, however, be stimulated to grow more actively by addition of IL-2. We therefore added to 5 U/ml of IL-2 to the HIV-producing cell line HUT $78/34_3$, to assess the influence of more active cellular growth on virus production. Within 3 days, a substantial increase of syncytia was seen in the culture. The RT-activity was monitored for 15 days in the virus-producing cultures with and without IL-2. As shown in Figure 3, the average RT-activity in the culture without IL-2 amounted to 15,000 cpm/ml SNF during this period. In the IL-2 containing culture the RT-activity increased until day 6 with a maximum RT-activity of 85,000 cpm/ml SNF ie, a 6-fold increase of RT-activity as a result of IL-2 addition. After this steep rise the RT-activity decreased and was no longer detectable on day 15. This decline was accompanied by death of the culture.

It has been reported that corticosteroids improve AIDS virus-isolation rates [Markham et al, 1986]. We have, therefore, investigated the effect of these hormones on HIV_{D34} replication in cord lymphocytes using end-point titration in presence of different hormone concentrations. In concentrations of 2.5–10 μg/ml [used by Markham et al, 1986] hydrocortisone had toxic effects on the target cells and no positive

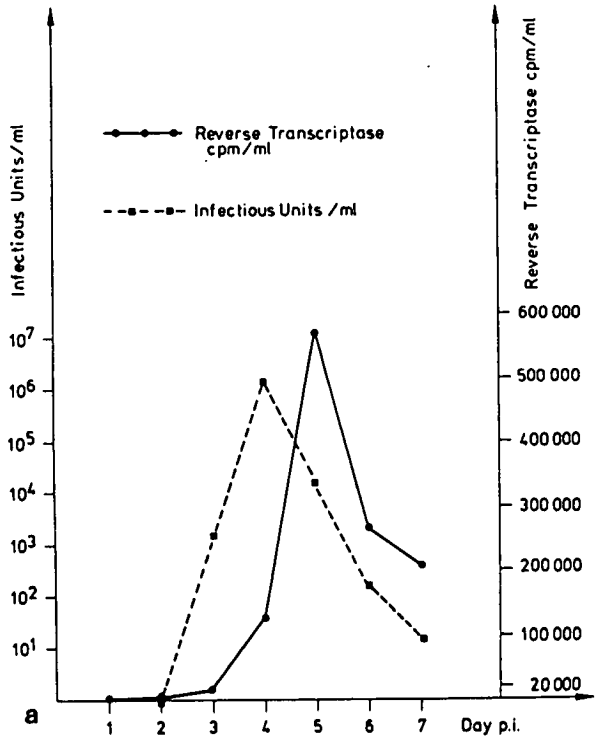

Fig. 2. Monitoring of a culture of HIV_{D34} for infectious units, RT-activity and CPE: (a) Titration of HIV_{D34} replication on a cord blood lymphocyte culture which was inoculated with 2×10^3 infectious units of virus on day 0 and monitored daily for RT-activity (see methods) and virus titre using the following technique: 5×10^5 freshly stimulated cord blood lymphocytes in 1 ml of medium were seeded into micro-titre wells. SNF of infected cultures was diluted 10^{-1} to 10^{-7}-fold and 100 μl from each titre step were inoculated per well. The wells were filled up to 1 ml with medium. After 24 hours each well was fed with 1 ml of medium. The following days 1 ml SNF was replaced by 1 ml of fresh medium. The endpoints were determined on day 6 by microscopic examination of the CPE. (b–d) Light microscopic photographs of culture described in (a): (b) uninfected control cells, (c) cells 3 days pi with HIV_{D34}, (d) cells 4 days pi with HIV_{D34}.

influence on virus propagation. In contrast, another glucocorticoide, dexamethasone, was not toxic in the range of 0.2–1.6 μg/ml, but an inoculum of at least 10-fold less virus could still be propagated in the culture containing the hormone as opposed to the control culture. In contrast to the positive effect of dexamethasone, polybrene did not increase the efficiency of virus detection in end-point titrations.

Taken together, all of the experiments described for this type-2 isolate demonstrate that its replication is strongly dependent on multiple factors of cell culture.

Molecular Cloning and Restriction Analysis of a Type-1 Isolate From Peripheral Blood Reveals Multiple Simultaneous Variants in One Patient

An ultimate aim of the analysis of HIV strains would be to correlate genetic changes in the strains with changes in their biological properties. To characterize one

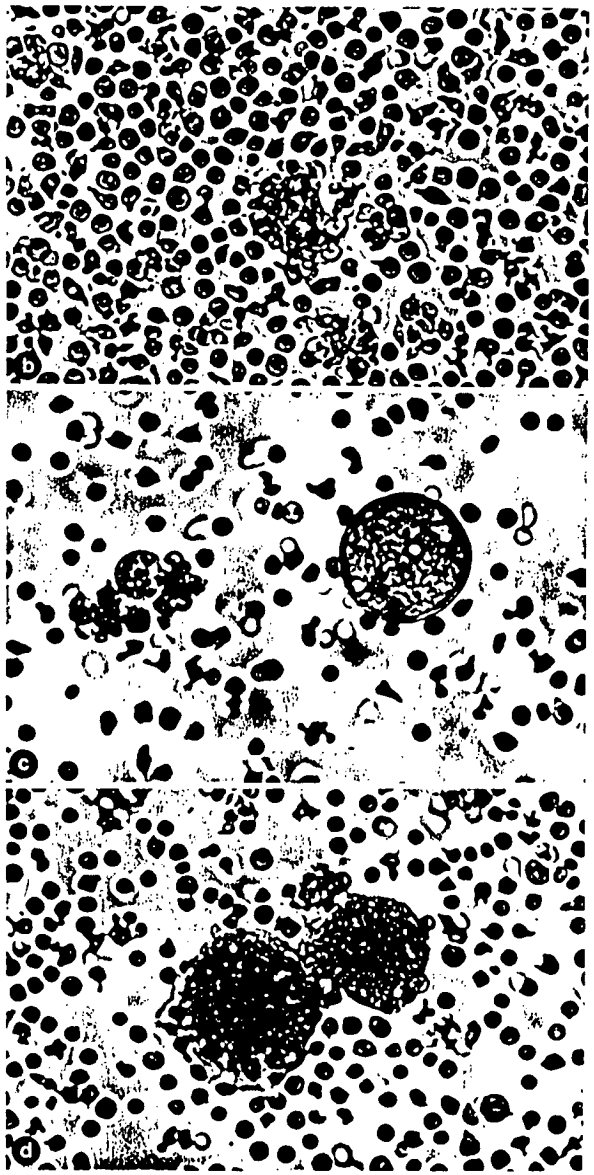

Fig. 2. (b-d)

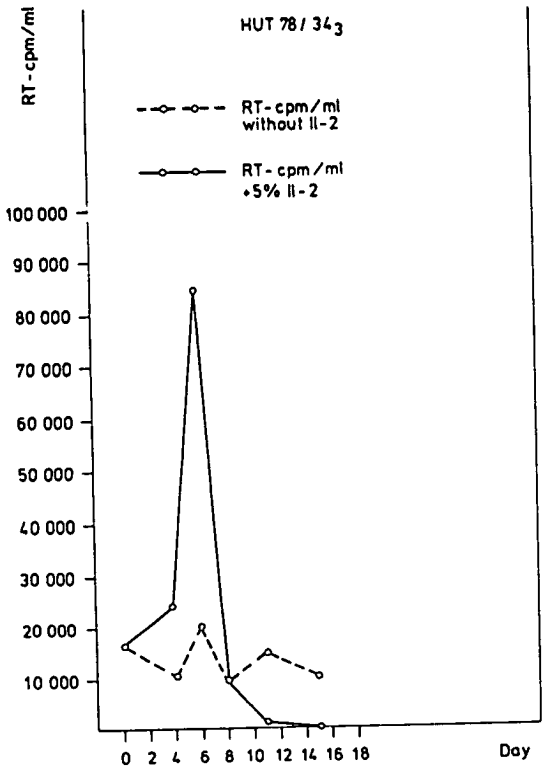

Fig. 3. Induction of virus replication by IL-2. Two sister cultures of HUT 78/34$_3$ -cells (permanently producing HIV$_{D34}$, 10 ml of cell suspension) at 1 × 10^6 cells/ml were used; 5 U/ml of Il-2 (Biotest, Frankfurt, FRG) were added to one culture on day 0. Every second day about 5 ml of the medium (supplemented with IL-2 for the culture to be grown in presence of IL-2) were replaced and the reverse transcriptase activity in the supernatant was monitored over a period of 15 days.

isolate of the most common HIV type 1 at the genetic level, isolate HIV$_{D31}$ [patient 3, Rübsamen-Waigmann et al, 1986] was molecularly cloned.

Southern blotting and hybridization of total cellular DNA from HIV$_{D31}$-infected cells with lambda BH 5/8-derived DNA had demonstrated that in patient 31 at least two polymorphic HIV-related viruses coexisted, which were characterized by 2 and 3 SstI sites, respectively [Rübsamen-Waigmann et al, 1986a]. By comparison with restriction data from other authors [Hahn et al, 1984; Muesing et al, 1985; Shaw et al, 1984; Hahn, 1985; Luciw et al, 1984] it was expected that two restriction sites correspond to one conserved cleavage site within the two LTRs, leading to a linear fragment of approximately 9kb, irrespective of the integrated or unintegrated form of viral DNA. The two other bands of approximately 5.5 kb and 3.5 kb could well fit to a single internal SstI site formerly described by Hahn et al [1984].

According to these results and following German safety regulations (ZKBS) the SstI digested fragments were cloned into lambda gt·WES, thus avoiding the cloning of complete LTR promotor regions and complete viral genomes. From screening

500,000 plaques three different clones were obtained from the env/lor region homologous to lambda BH 8 (HIV$_{D31}$, 4.1, HIV$_{D31}$, 8.1, and HIV$_{D31}$, 2.1). The two other clones (HIV$_{D31}$, 10.2 and HIV$_{D31}$, 1.1) represented two polymorphic sequences corresponding to lambda BH 5. The insert of 9 kb length present in HIV$_{D31}$-infected DNA digested with SstI was represented in a 6th clone of the genomic library but was lost upon subsequent propagation. The restriction patterns of all five clones differed remarkably from each other and from the patterns described form lambda BH5/BH8 (Fig. 4).

Thus, the data indicate that at the time of virus isolation, at least four different polymorphic virus DNAs coexisted in the patient (including the 9kb SstI variant not represented in the subclones). Because three out of three clones from the same genomic region of the virus differed from each other, it can be expected that a more extensive screening of a large lambda gene library would have resulted in an even larger collection of polymorphic variants.

As expected from the restriction analysis, at the nucleotide sequence level lambda HIV$_{D31}$, 2.1 also showed remarkable differences to lambda BH8. We sequenced 2,490 nucleotides and observed a 6.7% deviation from the lambda BH8 sequence due to point mutations and nine deletions/insertions, none of which destroyed the reading frame. The amino acid variation in the env/lor region of this clone is roughly 10% compared to lambda BH8 (unpublished data).

DISCUSSION

In this communication we have described properties of growth and cytopathogenicity of a large collection of HIV isolates as well as the genetic characterization of one of these strains.

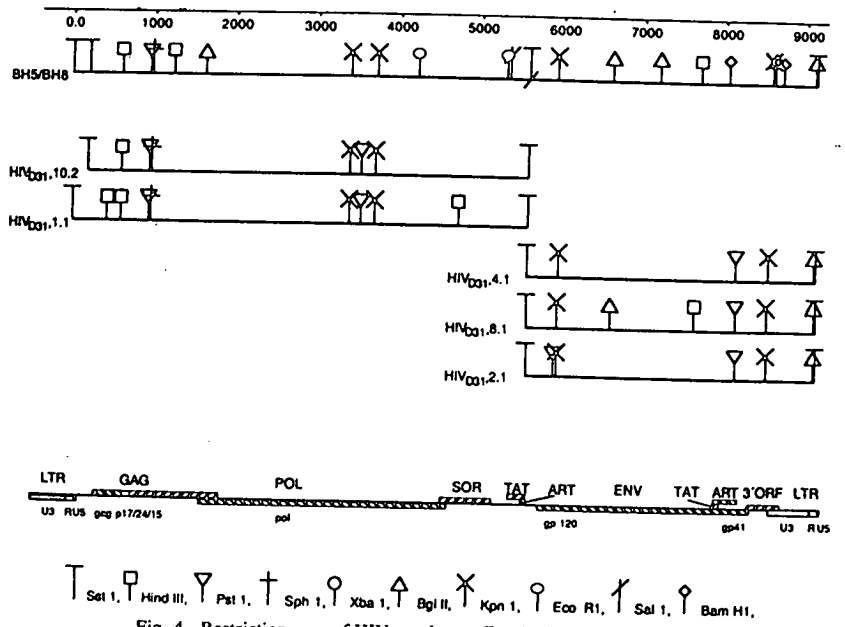

Fig. 4. Restriction map of HIV$_{D31}$ clones. For details, see text.

Clinical State, Isolation Frequency and Virus Type

In contrast to the observation of Gallo et al [1984], HIV was obtained more readily from diseased patients as compared with seropositive but clinically healthy individuals. One is therefore faced with the paradox finding that although fewer T4 target cells are present in the peripheral blood of these patients, more are infected and/or can be activated to produce virus.

In repeated attempts over a period of up to 1 year, it was impossible to isolate virus from some of the healthy seropositive patients. This finding seems to indicate that either there are latency periods in which virus expression is suppressed in infected individuals or that there are rare cases in which the virus could be eliminated from the body. However, in followup investigations on three healthy seropositive patients, from whom virus isolation was not possible originally, virus was isolated at a later time when their clinical status had deteriorated. From the available clinical data it was not possible to determine what was the event leading to the deterioration of the clinical status of these particular patients and leading to the activation of the apparently latent virus. Obviously, more work on patients carefully monitored clinically and virologically over extended periods of time is needed to understand the mechanism of virus latency and activation and their effect on the clinical picture.

It was reported recently [Asjö et al, 1986] that the type of HIV isolated was related to the clinical course of the patients. Most patients with overt AIDS were found to harbour a virus with a high replication rate (measured as RT-activity in the SNF of their PBL cultures) while patients with LAS or HIV-seropositives without symptoms were bearing a virus type with low replication rate. The result in the present investigation do not completely fall in line with this observation, although most AIDS patients were shown to be infected with viruses exhibiting strong CPE and good replication (type 1 or type 2). However, there were also several cases (about 20%), where a slowly growing virus (type 4) or a well-growing virus with little or no CPE (type 3) on lymphocytes was isolated from AIDS patients on repeated occasions.

The growth properties of HIV strains in lymphocytes is obviously only one biological parameter to characterize these viruses. The multiple pathological findings in AIDS patients and the fact that other important target cells exist for HIV, like the monocyte/macrophage, make it unlikely per se that simple correlations between growth properties of HIV on lymphocytes and clinical pictures exist.

Dependence of HIV Replication on Cellular Factors

The viruses isolated so far in our laboratory could roughly be grouped into four categories according to their growth properties in fresh peripheral lymphocytes. In all in vitro passages of the isolates, these characteristics remained stable. However, between these categories intermediate types were observed. Again, the growth on lymphocytes, although useful for characterization, is only one parameter. The full picture of biological variability of HIV will only emerge when all possible target cells are known.

It is speculated that differences in replication rates of the various HIV types were determined by differences in genes like tat and art [Sodroski et al, 1986]. The most cytopathogenic type-2 viruses might have been more or less independent of cellular factors for their replication. Even for one of these isolates [HIV_{D34}, patient 6 in Rübsamen-Waigmann et al, 1986a], which was characterized in more detail in this paper, we did, however, observe a marked dependency of its replication rate on external or cellular factors: 1) the virus grew much more slowly and to lower titres on an established lymphoma line as opposed to fresh peripheral lymphocytes, 2) its replication on the most permissive cell (the umbilical cord lymphocyte) could be enhanced further by addition of dexamethasone, and 3) incubation of the persistently infected low producing lymphoma cell HUT $78/34_3$ with IL-2 resulted in a markedly enhanced expression of the virus. Thus, in addition to virus-specific regulatory genes like tat and art, which activate replication, cellular factors are obviously very important in the control of replication, even for type-2 viruses.

Multiple Variants are Being Observed Within a Patient

The finding of several simultaneous variants in one patient raises the question if these variants are the results of multiple infections or of mutations which have taken place within this particular patient. There appears to be no reason to exclude the possibility of multiple infections: in early infections only a few of the total T4 cells are infected at any given time and more might get infected at a later stage. However, evidence is accumulating for continuous and rapid changes of HIV in an infected organism [Alizon et al, 1986; Spire et al, 1986; Shaw et al, 1986]. Rapid changes can also be observed from donor to recipient of an HIV infection: We have seen a case where the virus isolate from an HIV-infected mother was clearly a type-2 virus, while that of one of her 5-month-old twins was type 3, ie, hardly cytopathogenic for lymphocytes [Rübsamen-Waigmann et al, 1986b].

In contrast to the data presented in this communication Shaw et al [1986] observed only one particular restriction pattern of HIV in the isolate from a given patient at a given time. It seems unlikely that our finding is specific for patient 31. Preliminary results on clones obtained from isolates recovered from other patients also indicate the simultaneous presence of several different subtypes. The most likely explanation for the divergence between our observation and that of Shaw et al is a difference in the method of virus isolation. The isolation protocol used in the present study allows very rapid enrichment of HIV. We have so far molecularly cloned isolates, which were obtained within a maximum of 3 days from the patients blood. Thus, most likely the whole collection of virus subtypes which was originally carried by the patient was retained. By the use of other isolation protocols, however, where the inoculum is propagated on less susceptible cells for several weeks variants may get lost.

Apart from the fact that several HIV variants can coexist in a patient, there also appears to be a certain selection of variants for their target organs. As demonstrated in Table II, type-4 viruses were much more frequently isolated from CSF as compared to blood. When simultaneous attempts of virus isolation were made from blood and CSF of the same patient, the isolate from the blood often differed from that from the CSF in that it grew much better on lymphocytes. This was also observed for the CSF isolate from patient 31, from whom the peripheral isolate HIV_{D31} was obtained.

It has been assumed that the infection of the central nervous system does not occur via infected lymphocytes but that the virus is harboured by macrophage-derived cells like microglia cells. Furthermore, there is evidence in the literature that viruses which grow well on macrophages do not grow well on lymphocytes and vice verse [eg, Salahuddin et al, 1986]. Hence one explanation for the results mentioned above could be that out of a collection of virus subtypes entering the periphery not all reach

the central nervous system because not all are able to infect monocyte/macrophage-derived cells. Alternatively it could be assumed that the original virus undergoes mutations in the periphery or in the brain under the immune pressure of the organism [Clements et al, 1980], which optimize its growth in the host cells of its particular environment.

In conclusion, the data presented in this paper further substantiate the fact that a wide spectrum of HIV variants exist that differ not only in their restriction maps and nucleotide sequences but that mutations also result in substantially different biological properties. It becomes apparent that it will be impossible to understand the development of the complex disease AIDS before more knowledge is gained on the types of various target cells, on the mechanisms which control virus replication not only through viral genes, but also by cellular factors and on the function played by the immunological reaction of the host in the suppression and/or selection of variants within the patient.

The fact that stimulation of cells with IL-2 lead to enhanced expression of virus and death of a culture, which otherwise was in equilibrium between virus replication, cell lysis, and production of new cells, represents a warning for clinical trials, in which immunomodulating agents are being used. We are far from understanding all factors and cellular events that control the replication of HIV. Modulations of the immune system without concomitant antiviral therapy may well have the undesired effect of inducing additional virus growth.

ACKNOWLEDGMENTS

We would like to thank Dr. R.C. Gallo for the clones lambda BH5 and BH8. The excellent technical assistance of Ms. C. Arndt, Ms. S. Dohrmann, and Ms. U. Rapp is gratefully acknowledged. We thank Mr. H. Rupke for skillful preparation in electron microscopy. This work was supported by the Hessische Landesregierung, the Bundesminsterium für Forschung und Technologie, and the Verein zur Förderung der AIDS Forschung e. V Frankfurt.

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Journal of Medical Virology 23:67-73 (1987)

Isolation of Human Immunodeficiency Virus (HIV) From Plasma During Primary HIV Infection

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Human immunodeficiency virus (HIV) has been isolated from plasma in 6 of 7 patients showing clinical symptoms of a primary HIV infection. Parallel cultures from peripheral blood mononuclear cells (PBMC) yielded virus in 5 patients. In one case, virus could only be isolated from the cerebrospinal fluid but not from peripheral blood. Detectable viremia was transient and preceded the appearance of HIV specific antibodies. After cessation of acute symptoms, the frequency of HIV isolations was similar to that of asymptomatic carriers (23 and 26%, respectively). The role of the immune response in terminating detectable viremia remains to be established.

Key words: HIV, plasma, primary infection

INTRODUCTION

Seroconversion following infection with human immunodeficiency virus (HIV) has been reported to be associated with acute illness in some cases. A mononucleosislike disease was found to coincide with seroconversion in 9 out of 10 patients [Cooper et al, 1985]. By contrast in a study of 15 HIV-infected individuals, others found no signs of clinical illness associated with seroconversion [Weber et al, 1986]. It is not clear to which extent infected persons develop symptoms during the initial phase of the infection. The

Accepted for publication April 3, 1987.

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